Human CMV infection of endothelial cells induces an angiogenic response through viral binding to EGF receptor and β_1 and β_3 integrins

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Human cytomegalovirus (HCMV) infection is associated with atherosclerosis, transplant vascular sclerosis, and coronary restenosis. A common theme in these vascular diseases is an increased rate of angiogenesis. Angiogenesis is a complex biological process mediated by endothelial cell (EC) proliferation, migration, and morphogenesis. Although angiogenesis is a normal process in the host, its dysregulation, after viral infection or injury to the vessel wall, is associated with plaque development in atherosclerotic patients. We now document that HCMV infection results in increased EC proliferation, motility, and capillary tube formation. The observed HCMV-induced angiogenic response depended on viral binding to and signaling through the β_1 and β_3 integrins and the epidermal growth factor receptor, via their ability to activate the phosphatidylinositol 3-kinase and the mitogen-activated protein kinase signaling pathways. Because a proangiogenic response drives the neovascularization observed in atherosclerotic disease, our findings identify a possible mechanism for how HCMV infection contributes to vascular disease.

angiogenesis | cardiovascular disease | pathogenesis | receptor–ligand-mediated signaling

Cardiovascular diseases (CVDs) are leading causes of death in developed countries, with complications from atherosclerosis accounting for a majority of these deaths (1). Although multiple risk factors, including hypertension and hypercholesterolemia, are linked to CVD development, up to 50% of patients with atherosclerosis do not portray these typical risk factors (2). One such risk factor attracting recent attention is vascular inflammation, caused by vessel wall injury and endothelial cell (EC) dysfunction (2, 3) and triggered by infectious agents such as human cytomegalovirus (HCMV) (4–6).

ECs separate the blood from surrounding tissue and are important in the maintenance of existing vessels and the formation of new vessels (7). Angiogenesis is a normal process mediated by the proliferation, migration, and morphogenesis of ECs from preexisting vessels (8). Chronic injury to a vessel wall, however, can promote aberrant angiogenic responses, causing the plaque neovascularization observed in CVD patients (8–10). Although vascular injury and EC dysfunction are critical for this plaque neovascularization (8–10), the events prompting these vascular changes are not clearly identified. Infection with pathogens, such as HCMV, is thought to be a primary factor in this type of vascular damage (4–6).

HCMV establishes a life-long persistent infection in the human host (11). In immunocompetent hosts, although infection is generally mild (11), there now is mounting evidence that correlate HCMV infection with the development/severity of CVDs (4–6). HCMV seropositivity correlates with a significant increase in the risk of coronary artery disease (4–6). Seropositivity also correlates with increased severity of atherosclerosis in transplanted hearts and rates of graft rejection (4–6). In animal models, rodent CMV infection exacerbates atherosclerotic lesion development and accelerates transplant vascular sclerosis leading to graft rejection (4, 5).

HCMV also is a primary candidate in the etiology of CVDs because the cells associated with CVDs are primary *in vivo* cellular targets for HCMV (2, 4–6). The mechanisms by which HCMV infection contributes to CVDs remain unresolved. We propose that infection of ECs initiates an inflammatory response through the aberrant activation of the endothelium resulting in inflammatory cell recruitment and plaque neovascularization. Supporting this possibility is our recent work showing that HCMV infection of ECs induced a proinflammatory response resulting in naïve monocyte recruitment to the infected endothelium (12). Additional reports document that HCMV infection alters EC function by increasing proinflammatory cytokine production, increasing leukocyte adhesion molecules expression, disrupting EC integrity, and impairing antithrombogenic activity (4–6). Because HCMV results in lifelong persistence in the endothelium (11, 13), a pathogenic consequence would be chronic activation and long-term dysregulation of the endothelium.

We put forward the idea that these viral-induced cellular changes directly contribute to the vascular diseases associated with HCMV infection. Furthermore, because these EC changes occur during angiogenic responses (8–10), we hypothesized that HCMV infection of ECs triggers an angiogenic response. We show here that EC proliferation, migration, and morphogenesis, hallmarks of angiogenesis and CVD development, occur after infection. We found these changes depended on viral binding to the epidermal growth factor receptor (EGFR) and the β_1 and β_3 integrins, reported receptors for HCMV infection (14–16) and regulating angiogenesis (17, 18). Mechanistically, HCMV-induced angiogenesis depended on the activities of EGFR and Src family tyrosine kinases, along with the activation of the downstream phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Because EGFR is a "nontraditional" angiogenic receptor mediating pathogenic angiogenesis within the host (19–21), these data provide insight into a mechanism linking HCMV infection to CVDs and define that the receptors used for viral infection likely initiate the pathology observed in infected individuals.

Results

HCMV Infection Promotes an Angiogenic Response. We documented that HCMV infection of ECs increased the expression of cell adhesion molecules, naïve monocyte recruitment, and EC permeability while concomitantly degrading lateral junction pro-

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The authors declare no conflict of interest.

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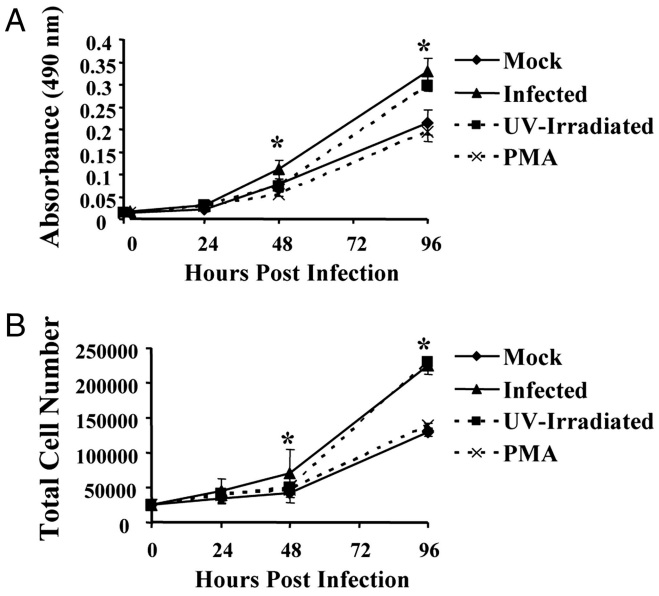

Fig. 1. HCMV infection enhanced EC proliferation. Proliferation assays were performed on mock-infected, HCMV-infected, UV-irradiated HCMV-treated, or PMA-treated HMECs. An MTS assay (*A*) and total cell counts (*B*) were performed at various times after infection. Results are plotted as the mean ± SD with significant differences of *, $P < 0.05$.

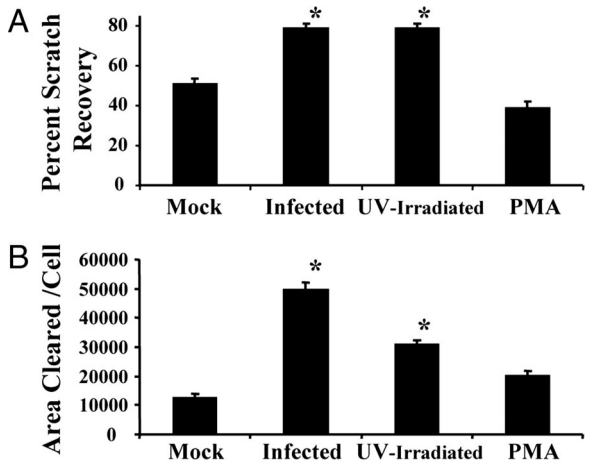

Fig. 2. HCMV infection increased EC motility. HMECs were mock-infected, HCMV-infected, UV-irradiated HCMV-treated, or PMA-stimulated for 12 h. (*A*) Scratch assays were performed, and the average percentage scratch recovery was determined. (*B*) Phagokinetic motility tract assays were performed, and the average area of colloidal gold cleared per cell was determined. Results are plotted as the mean ± SD with significant differences of *, $P < 0.05$.

teins (12). These HCMV-mediated EC changes typically are observed during angiogenesis (2, 17). Therefore, we asked the question: Does HCMV infection directly induce an angiogenic response? During angiogenesis, ECs proliferate, migrate to the site of damage and/or inflammation, and morph to form new capillary tubes (8, 17, 18).

We first investigated whether HCMV promoted EC proliferation using two distinct assays: (*i*) a MTS assay (Figs. 1*A*) and (*ii*) a total cellular number count (Fig. 1*B*). Viral-enhanced proliferation was observed as early as 24 h postinfection (hpi) and was significantly increased ($P < 0.05$) by 48 hpi (Fig. 1). By 96 h, mock-infected human microvascular ECs (HMECs) went through an average of two population doublings, whereas HCMV-infected HMECs went through an average of more than three population doublings, diverging from studies performed in fibroblasts, where HCMV has been documented to prevent cellular proliferation (22). HMECs treated with UV-irradiated HCMV also proliferated at a faster rate than mock-infected cells. Infection of HMECs with a range of multiplicities of infection (MOIs; 0.01–20) demonstrated that HCMV-induced EC proliferation occurred at a slower rate when lower MOIs were used to infect cells [supporting information (SI) Fig. 8]. Thus, viral-induced EC proliferation occurs at multiple MOIs and is not specific to only a high MOI. Furthermore, the results hint that different threshold levels of signaling in ECs may control different rates of proliferation. We also observed a significant increase in umbilical vein ECs in the S and G_2/M phases of the cell cycle by 48 and 72 hpi when compared with mock-infected cells (data not shown), providing further support for the enhanced proliferation we observed. Phorbol 12-myristate 13-acetate (PMA), a known angiogenic promoter (23), did not stimulate enhanced HMEC proliferation in our system.

In addition to proliferating, individual ECs also need to migrate to the site of vascular damage and/or to where new capillary tubes are to be formed. To investigate whether HCMV infection promoted EC motility, a scratch assay was performed on a confluent monolayer of HMECs (Fig. 2*A*; see SI Fig. 9*A* for images). The percentage scratch recovery was calculated at 12 hpi, a time when no significant differences in infected versus mock-infected HMEC cell numbers were observed (Fig. 1). All initial scratches were similar. By 12 hpi, HCMV-infected and UV-irradiated HCMV-treated HMECs showed a significant recovery ($P < 0.05$) of the initial scratch (≈80%), whereas mock-infected or PMA-stimulated cells recovered only 50% of the initial scratch. Second, phagokinetic random motility assays were performed to examine individual EC motility (Fig. 2*B*; see SI Fig. 9*B* for images). HCMV-infected and UV-irradiated HCMV-treated HMECs showed a significant ($P < 0.05$) 3- to 5-fold increase in motility compared with their mock counterparts (Fig. 2*B*). Last, ECs must be able to form new capillary tubes and blood vessels. Tubular morphogenesis assays revealed a significant ($P < 0.05$) 2- to 2.5-fold increase in the number of branches/capillary tubes occurred after HCMV infection, UV-irradiated HCMV treatment, or PMA treatment of HMECs (Fig. 3; see SI Fig. 10 for images).

ECs from different vascular beds could have different responses after infection (12); thus, we investigated whether HCMV infection of human macrovascular aortic ECs (HAEC) induced an angiogenic response. HCMV infection of HAECs increased cellular proliferation, motility, and morphogenesis similar to that seen with HMECs (data not shown). Together, these findings provide strong evidence that HCMV infection of ECs promotes the three hallmarks of angiogenesis. Because these changes occurred in the absence of new viral gene expression, the findings identify that receptor ligand-mediated signaling may be the key determinant triggering these EC changes.

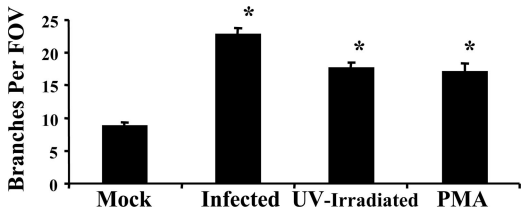

Fig. 3. HCMV infection promoted capillary tube formation. HMECs were mock-infected, HCMV-infected, UV-irradiated HCMV-treated, or PMA-stimulated and plated onto Matrigel for 12 hpi, and then the average number of branches was determined. Results are plotted as the mean ± SD for each group with significant differences of *, $P < 0.05$.

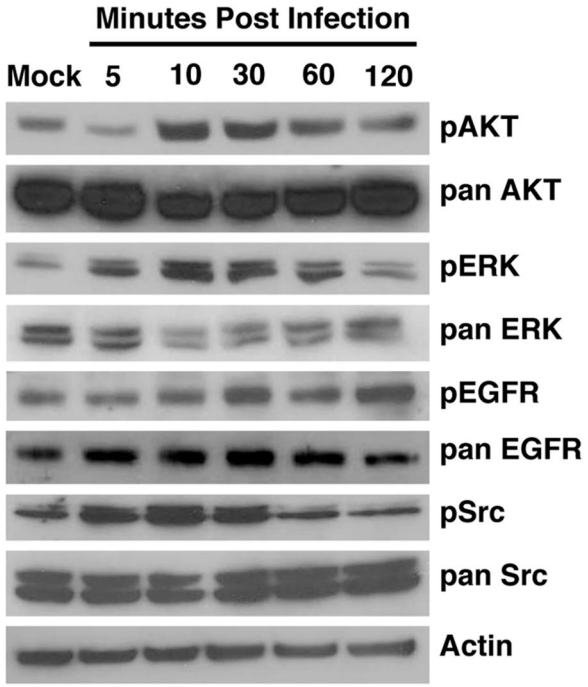

Fig. 4. HCMV infection rapidly activated ECs. HMECs were mock-infected or HCMV-infected for 0 min (mock infection) out to 120 min. Western blot analyses of pan EGFR, pEGFR, pan AKT, pAKT, pan Src, pSrc, pan ERK, pERK, and actin were performed with equal protein loading of each sample.

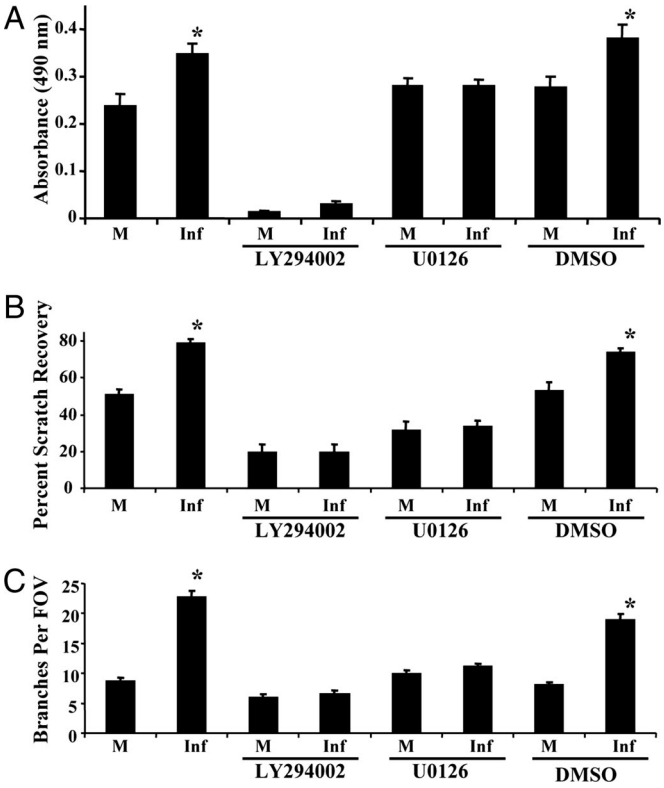

Fig. 5. The PI3K and MAPK pathways are required for the HCMV-induced angiogenic response. HMECs were mock-infected or infected with HCMV after pretreatment with LY294002, U0126, DMSO, or no pretreatment. MTS (only the 96-hpi point is shown) (*A*), scratch motility (*B*), and morphogenesis (*C*) assays were performed. Results are plotted as the mean ± SD for each group with significant differences of *, $P < 0.05$.

PI3K and MAPK Signaling Mediates the Observed Angiogenic Response. The regulation of EC function is controlled by outside-in signals (17, 18). The PI3K and MAPK pathways are two pathways involved in angiogenesis (17, 18) that also are activated by HCMV [in fibroblasts and monocytes (14, 15, 24, 25)]. To examine whether HCMV infection stimulated these pathways in ECs, Western blot analyses to examine levels of phosphorylated AKT (pAKT) and ERK (pERK) were performed on confluent, serum-starved mock-infected or HCMV-infected HMECs (Fig. 4). Between 10 and 60 min postinfection (min pi), levels of pAKT and pERK1/2 increased in infected cells when compared with levels observed in mock-infected ECs. By 120 min pi, levels of pAKT and pERK1/2 in HCMV-infected HMECs returned to that seen in mock-infected cells. No dramatic changes were seen in pan AKT or ERK levels. UV-irradiated HCMV-treated HMECs showed similar kinetics of PI3K activation to that of wild-type HCMV (data not shown).

To examine the role of HCMV-induced PI3K and MAPK activation on the observed angiogenic response, we used LY294002, to inhibit PI3K activity, and U0126, to inhibit the MAPK-ERK kinase (MEK1/2). Both drugs are specific for their respective pathways and nontoxic at the concentrations used (14, 24, 26). As described above, HCMV infection significantly ($P < 0.05$) increased HMEC proliferation, motility, and morphogenesis (Fig. 5 *A–C*), a response that was significantly ($P < 0.05$) abrogated when cells were treated with LY294002 or U0126 before infection. The solvent control (DMSO) had no effect on the HMEC response when added before infection (Fig. 5 *A–C*). The role these pathways played in the regulation of basal levels of EC function varied depending on the EC function examined. Regardless of the varying role these pathways play in basal levels of EC function, these data show HCMV-induced PI3K and MAPK activation is required for viral-mediated angiogenesis.

EGFR and Src Family Tyrosine Kinases Regulate the Induced Angiogenic Response. Multiple receptors, including growth factor receptors (EGFR) and integrins ($\alpha_v\beta_3$ and $\alpha_2\beta_1$), have been implicated in promoting angiogenesis (17, 18). These same receptors are reported to be required for HCMV entry, infection, and receptor-mediated signaling events in fibroblasts (14–16), suggesting a possible link between infection and the pathogenic consequences of infection of ECs. To decipher whether these cellular receptors, upstream of the PI3K and MAPK pathways, are responsible for triggering the switch to an angiogenic phenotype after infection, we performed Western blot analyses to examine pan and phosphorylated EGFR (pEGFR) and Src (pSrc) levels in confluent, serum-starved mock-infected or HCMV-infected HMECs (Fig. 4). The levels of pEGFR and pSrc increased in infected cells between 5 and 30 min pi. EGFR tyrosine kinase activation remained throughout the experiment, whereas Src activation was transient, returning to undetectable levels by 120 min pi. No dramatic changes were seen in pan EGFR or Src levels.

Next we used inhibitors of the EGFR tyrosine kinase (AG1478) and the Src family of tyrosine kinases (PP2) to show that these kinases functionally regulated the viral-mediated EC response. Both compounds are potent, specific inhibitors (14, 15) that are nontoxic at the concentrations used. As described above, infected HMECs and DMSO-treated, HCMV-infected HMECs proliferated (Fig. 6*A*), migrated (Fig. 6*B*), and formed capillary tubes (Fig. 6*C*) at significantly ($P < 0.05$) higher levels than their uninfected counterparts. Pretreatment with AG1478 or PP2 before infection significantly ($P < 0.05$) abrogated HCMV-induced HMEC proliferation and motility (Fig. 6 *A* and *B*). In contrast, pretreatment of HMECs with only AG1478 before infection significantly inhibited ($P < 0.05$) HCMV-mediated morphogenesis (Fig. 6*C*). Mock-infected HMECs pretreated

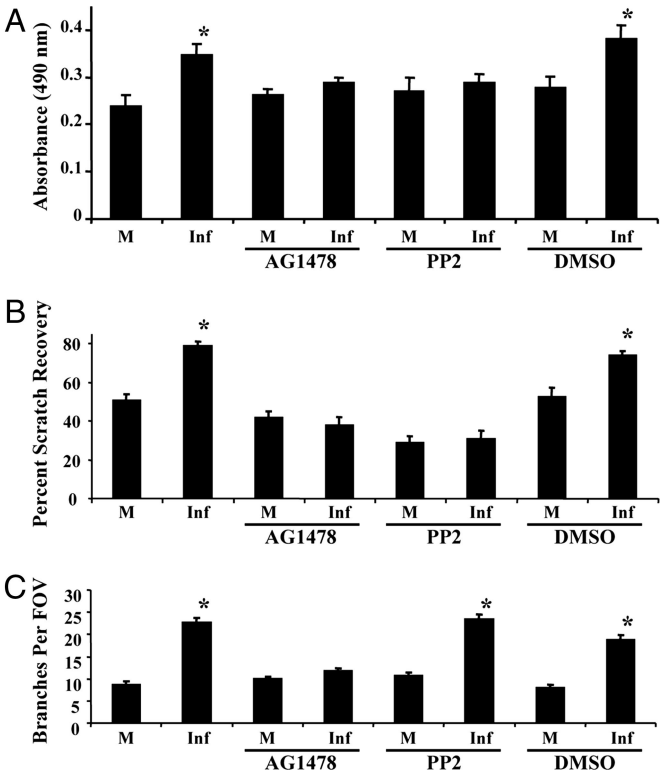

Fig. 6. The EGFR tyrosine kinase was required for the HCMV-induced angiogenic response, whereas the Src family of tyrosine kinases was only partially required. HMECs were mock-infected or HCMV-infected after pretreatment with AG1478, PP2, DMSO, or no pretreatment. MTS (only the 96-hpi point is shown) (*A*), scratch motility (*B*), and morphogenesis (*C*) assays were performed. Results are plotted as the mean ± SD for each group with significant differences of *, $P < 0.05$.

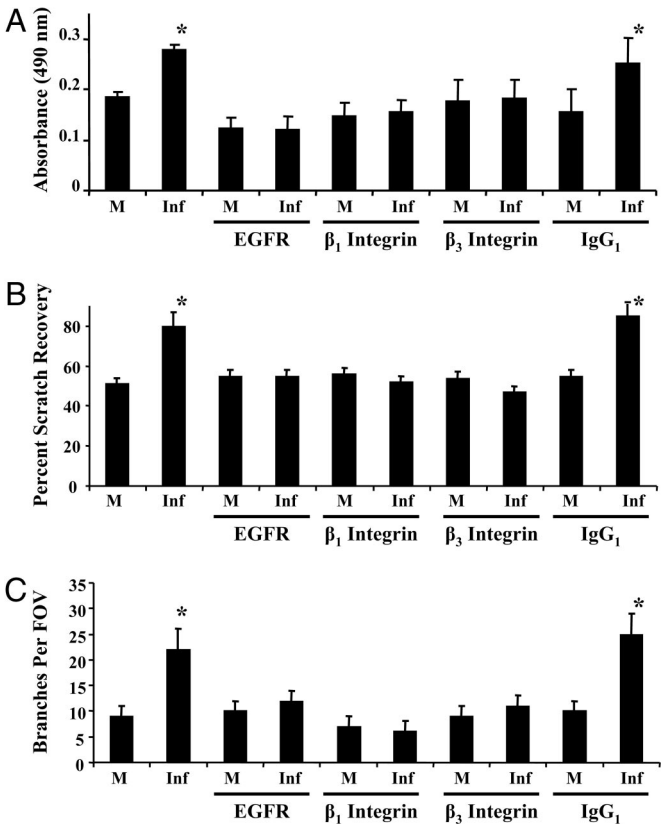

Fig. 7. Viral binding to EGFR and the β_1 and β_3 integrins dictate the HCMV-induced angiogenic response. HMECs were pretreated with function-blocking antibodies to EGFR, the β_1 and β_3 integrins, or the isotype control for 1 h before mock or HCMV infection. MTS (only the 96-hpi point is shown) (*A*), scratch motility (*B*), and morphogenesis (*C*) assays were performed. Results are plotted as the mean ± SD for each group with significant differences of *, $P < 0.05$.

with AG1478 or PP2 exhibited similar biological changes as untreated or DMSO-pretreated HMECs (Fig. 6 *A–C*). Together, these results suggest HCMV-induced activation of the EGFR tyrosine kinase was required for each of the viral-mediated angiogenic steps, whereas the activation of Src family tyrosine kinases was required only for HCMV-induced EC proliferation and motility.

Viral Binding to EGFR and the β_1 and β_3 Integrins Was Required for the Induced Angiogenic Response. The data described above represent the initial finding that EGFR-mediated and integrin-mediated signaling triggers HCMV-induced EC changes. The direct role these receptors play in potential pathogenic cellular changes after direct binding of HCMV to ECs, however, has not been addressed. Previous work showed that HCMV entry into fibroblasts required viral binding to EGFR, $\alpha_v\beta_3$ integrin, $\alpha_2\beta_1$ integrin, and $\alpha6\beta_1$ integrin (14–16). Of these cellular receptors, only EGFR, $\alpha_v\beta_3$ integrin, and $\alpha_2\beta_1$ integrin have been shown to be important in regulating angiogenesis (17, 18), suggesting their engagement during viral binding may be a key determinant in viral-mediated changes in ECs. It should be noted that the role EGFR plays in HCMV infection is controversial as a recent study reported that EGFR is not required for HCMV infection of fibroblasts and a single EC line (27), although the authors used a laboratory strain that lacks an intact U_LB' region like the clinical/clinical-like isolates used here. Regardless, we find that EGFR-mediated and integrin-mediated signaling (via Src) is rapidly activated in ECs after infection (Fig. 4), suggesting their engagement after viral binding is intimately associated with functional changes in ECs. Therefore, we focused on the role viral binding to EGFR and the β-subunits of various integrins (β_1–β_4) played in HCMV-induced angiogenesis because of their role in regulating angiogenesis and with the exception of the β_2 integrins, are expressed on ECs (17, 18).

Using function-blocking antibodies to inhibit viral binding to specific cellular receptor (EGFR or the β_1–β_4 integrins) or an isotype-matched IgG_1 control antibody, we found that viral binding to EGFR and the β_1 and β_3 integrins was required for HCMV-induced angiogenesis. HCMV infection significantly ($P < 0.05$) increased HMEC proliferation (Fig. 7*A*), motility (Fig. 7*B*), and morphogenesis (Fig. 7*C*), which also occurred when infected HMECs were pretreated with function-blocking antibodies to the β_2 and β_4 integrins (SI Fig. 11*A*) or the isotype control (Fig. 7 *A–C*). When HMECs were pretreated with function-blocking antibodies specific for EGFR or the β_1 or β_3 integrins before infection, HMEC proliferation (Fig. 7*A*), motility (Fig. 7*B*), and morphogenesis (Fig. 7*C*) were significantly inhibited ($P < 0.05$) and returned to that observed in mock-infected cells, showing that viral binding to these receptors was required for the HCMV-induced angiogenic response. HMECs also were pretreated with function-blocking antibodies to vascular EC growth factor receptor 1 (VEGFR-1) and VEGFR-2 before infection. The HCMV-induced angiogenic response was not altered by the presence of the VEGFR-1 and VEGFR-2 neutralizing antibodies (SI Fig. 11*B*). When HMECs were pretreated with any of these antibodies and mock-infected, there was no significant change in EC function (Fig. 7 *A–C*). Together,

these findings identify that HCMV binding to specific cognate receptors directly triggers an angiogenic response.

Discussion

We show here that direct HCMV infection of ECs induces a bona fide angiogenic response, offering insight into a biological link between HCMV infection and viral-mediated vascular diseases. But why would the virus produce a proangiogenic response? We attribute these cellular changes to the HCMV strategy required for hematogenous dissemination after primary infection, the seeding of virus to peripheral organs, and the establishment of viral persistence (12, 28). Unfortunately, these HCMV-induced cellular changes critical for viral survival also result in pathogenic consequences, establishing the paradigm that the strategy HCMV uses to survive within the host is directly related to viral-mediated diseases.

We observed that viral-binding to the cellular surface was sufficient to promote the observed angiogenic response. It is known that HCMV infection of fibroblasts occurs via viral binding to the cell surface receptors EGFR (14, 15), $\alpha_v\beta_3$ integrin (14, 16), $\alpha1\beta_1$ integrin (16), $\alpha6\beta_1$ integrin (16), and toll-like receptor 2 (29). We proposed that it was these same receptor–ligand interactions shown in fibroblasts that linked HCMV infection to viral-induced vascular pathologies. Of note, the cellular receptors, EGFR and the $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins can regulate angiogenesis (17, 18). With our data, we conclude that direct HCMV binding to EGFR and the β_1 and β_3 integrins serves as a true trigger for HCMV-induced changes in ECs.

HCMV binding to the β_1 and the β_3 integrins was necessary for HCMV-induced morphogenesis, yet integrin-mediated signaling was not required for this response. This seemly contradictory finding likely stems from the observation that viral binding to the integrins is required for EGFR-mediated signaling (14). Therefore, the data suggest that although integrin-mediated signaling was not required for HCMV-induced capillary tube formation, viral binding to the β_1 and β_3 integrins was critical for coordinated signaling with EGFR. Our data also identifies that viral receptor/ligand signaling during entry is distinct in different cell types. EGFR activation is transient in fibroblasts (14, 15), but as we show is sustained in infected HMECs, suggesting HCMV-mediated EGFR activation in ECs may be chronic, consistent with the observed long-term HCMV-mediated changes in EC function. Perhaps these differences in signaling explain the enhancement of cellular proliferation, with complete cell-cycle progression, we see in infected ECs versus the cell-cycle blocks observed in infected fibroblasts (22). Although at present the mechanisms for the differences in cell-type-dependent signaling is unknown, it likely stems from the differences in the biological characteristics of the different cell types, along with different viral proteins required for HCMV infection of ECs versus infection of fibroblasts (30, 31). Nevertheless, although we have not examined the long-term consequences of infection on changes in EC function, we speculate that HCMV only induces an early proliferative burst that coincides with the early activation of ECs before shifting toward a more committed differentiated phenotype that allows for stable tube formation. Collectively, the data suggest that although a set series of cellular receptors are used for infection of multiple cell types (25), the outcome of viral infection after receptor/ligand signaling is distinct in different cell types.

VEGFR-1 and VEGFR-2 are key cellular receptors that trigger angiogenesis through the activation of the PI3K and MAPK pathways (17), but we saw no role for viral binding to these receptors in HCMV-mediated angiogenesis. The hepatocyte growth factor receptor, another receptor that induces an angiogenic response (17) through the PI3K and MAPK pathways, also was not activated in infected ECs (data not shown). In addition, function-blocking antibodies specific for the β_4 integrins, which have a role in regulating angiogenic responses (18), had no effect on the HCMV-mediated response. Furthermore, the Ras superfamily of small GTPases, which can be activated via integrin-mediated signaling to promote an angiogenic response (17, 18), did not appear to be involved in HCMV-induced angiogenesis (data not shown). We also stimulated cells with PMA, a proangiogenic agent that induces EC tubular morphogenesis (23). In our system, PMA had both a proangiogenic (induced HMEC morphogenesis) and an antiangiogenic (not sufficient to promote HMEC proliferation and motility) effect on ECs. These findings add support to the idea that although a multitude of cellular receptors and signaling components contribute to normal angiogenic responses, the HCMV-induced dysregulation of this process is unique and mediated through the specific binding of HCMV to EGFR and the β_1 and β_3 integrins and the downstream activation of the PI3K and MAPK pathways.

Activation of VEGFR-2 is considered to be the main event triggering angiogenic responses in adults (17), and EGFR-mediated signaling has been linked to pathogenic angiogenesis—for example, in tumor growth (17, 19–21)—and now is a major pharmacological target for inhibiting tumor growth (19–21, 32). Therefore, we propose that HCMV infection of ECs utilizes a nontraditional angiogenic receptor and, in so doing, initiates a pathogenic angiogenic response. Together, we suggest that viral binding is the central determinant for the required early steps in viral-mediated vascular changes, although other viral proteins such as receptor homologues and G protein-coupled receptors also likely contribute to disease progression. Because angiogenesis promotes plaque neovascularization and inflammatory cell infiltration into plaques (8–10), we propose our findings offer a plausible mechanism for the initiation of the vascular abnormalities observed in infected patients. The observation that viral-mediated changes occur across a spectrum of MOIs, with higher MOIs showing a more dramatic effect, provides additional support for pathogenesis *in vivo*. In healthy hosts, where HCMV infection is associated with long-term atherosclerotic disease, low viral loads after primary infection would be expected to cause an initial vascular insult and then the continued low-level reactivation of latent virus over the life of the host would be expected to set up the chronicity of the viral-mediated vascular disease. However, high viral loads are seen in immunocompromised patients, where HCMV infection causes more rapid and severe vascular disease such as transplant vascular sclerosis.

Materials and Methods

Cell and Virus Cultures. HMECs and HAECs were cultured in endothelial growth medium (EGM; Clonetics). HCMV TB40/E-UL32 and Towne/E were cultured in fibroblasts and gradient-purified (12). UV-inactivated virus was prepared from gradient-purified virus (28) and did not express detectable IE gene products in fibroblasts (data not shown). ECs were infected at MOIs ranging from 0.01 to 20 based on infectivity of fibroblasts or mock-infected with media alone. One hundred percent of ECs were infected with both viruses using a MOI of 10–20 (12). Both viruses were used throughout the study with similar results; results from experiments with the TB40/E-UL32 virus are shown.

Proliferation Assays. MTS assays (Promega) were performed by using the manufacturer's protocol. HMECs were grown to confluence, serum-starved for 24 h, and treated with preconditioned EGM for 24 h. Cells were harvested in preconditioned EGM, and 1×10^3 cells were replated at subconfluent levels before mock infection, HCMV infection (MOI 0.01–20), or UV-irradiated HCMV treatment (equivalent MOI 20) (12). Total cell count assays were performed initially with 2.5×10^4 HMECs treated as described above. Assays were performed at the beginning of the experiment out through 96 hpi.

In some experiments, HMECs were mock-infected or infected with HCMV (MOI 20) after a 45-min incubation with LY294002 (50 μM; Promega), U0126 (10 μM; Calbiochem), PP2 (1 μM; Calbiochem), AG1478 (1 μM; Calbiochem), or DMSO (Sigma). In other experiments, ECs were incubated with function-blocking antibodies (from Chemicon and R&D Systems specific for EGFR (1

μg/ml), the β_1–β 4 integrins (20 μg/ml), VEGFR-1 and VEGFR-2 (20 μg/ml), or the isotype-matched control antibody (20 μg/ml) for 1 h at 4°C before being plated and mock-infected or infected with HCMV (MOI 20).

Motility Assays. HMECs were grown to confluence, serum-starved for 24 h, and treated with preconditioned EGM for 24 h. For the *in vitro* scratch assay (33), cells were injured with a sterile pipet tip, washed, and treated as described above. Immediately after infections and at 12 hpi, images of the scratches were captured for five random fields of view (FOV) along the scratch. By using ImageJ software, EC migration from the edge of the injured monolayer was quantified and the average scratch width was determined. Motility assays also were performed using the inhibitory drugs and function-blocking antibodies described above. For the phagokinetic motility assay (24), cells as treated above were harvested in preconditioned EGM, and 1×10^3 cells were plated on colloidal gold-covered coverslips. At 12 hpi, cells were fixed and mounted in glycerol on slides. Track images of individual cells were captured at $\times$100 magnification, and the average area cleared per cell was determined by using Scion Image software.

Capillary Tube Formation Assay (34). Twenty-four-well plates were coated with growth factor-reduced Matrigel (BD Biosciences). HMECs were grown to confluence, serum-starved for 24 h, and treated with preconditioned EGM for 24 h. ECs were harvested in preconditioned EGM, and 5×10^4 HMECs were added to each well and treated as described above. At 12 hpi, images of capillary tube formation were captured at $\times$100 magnification. Fifteen random FOV were captured per treatment, and the average number of branches per FOV was determined. Morphogenesis assays were performed by using the inhibitory drugs and function-blocking antibodies described above. Apoptotic bodies were never observed in any of the different arms of the experiment.

Western Blot Analysis. Cell lysates were harvested in Laemmli buffer (Bio-Rad). Equal sample amounts were separated by SDS/PAGE and transferred to PVDF membranes (Bio-Rad). After incubation with primary antibodies, blots were washed, incubated with horseradish peroxidase-conjugated secondary antibodies, and washed again. Protein was detected by Enhanced Chemiluminescence Plus (Amersham) following the manufacturer's protocol. Primary and secondary antibodies were obtained from Santa Cruz Biotechnology and Cell Signaling.

Statistical Analysis. All experiments were performed in triplicate, and results are plotted as mean $\pm$ SD. The statistical significance between experimental means was determined with Student's *t* test, and differences of $P < 0.05$ were considered significant.

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1. Murray CJ, Lopez AD (1997) Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet* 349:1436–1442.
2. Epstein SE, Zhou YF, Zhu J (1999) Infection and atherosclerosis: Emerging mechanistic paradigms. *Circulation* 100:e20–e28.
3. Libby P, Ridker PM, Maseri A (2002) Inflammation and atherosclerosis. *Circulation* 105:1135–1143.
4. Streblow DN, Orloff SL, Nelson JA (2001) Do pathogens accelerate atherosclerosis? *J Nutr* 131:2798S–2804S.
5. Streblow DN, Dumortier J, Moses AV, Orloff SL, Nelson JA (2008) in *Current Topics in Microbiology and Immunology: Human Cytomegaloviruses*, eds Stinski MF, Shenk T (Springer, Berlin), pp 397–415.
6. Söderberg-Nauclér C (2006) Does cytomegalovirus play a causitive role in the development of various inflammatory diseases and cancer? *J Intern Med* 259:219–246.
7. Sumpio BE, Riley JT, Dardik A (2002) Cells in focus: EC. *Int J Biochem Cell Biol* 34:1508–1512.
8. Lip GY, Blann AD (2004) Thrombogenesis, atherogenesis and angiogenesis in vascular disease: A new 'vascular triad'. *Ann Med* 36:119–125.
9. Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS (2001) Atherosclerosis and cancer: Common molecular pathways of disease development and progression. *Ann NY Acad Sci* 947:271–293.
10. Moulton KS (2001) Plaque angiogenesis and atherosclerosis. *Curr Atheroscler Rep* 3:225–233.
11. Mocarski ES, Jr, Shenk T, Pass RF (2007) in *Fields Virology*, eds Knipe DM, Howley PM (Lippincott Williams & Wilkins, Philadelphia), pp 2701–2772.
12. Bentz GL, Jarquin-Pardo M, Chan G, Smith MS, Sinzger C, Yurochko AD (2006) Human cytomegalovirus (HCMV) infection of ECs promotes naïve monocyte extravasation and transfer of productive virus to enhance hematogenous dissemination of HCMV. *J Virol* 80:11539–11555.
13. Fish KN, Soderberg-Naucler C, Mills LK, Stenglein S, Nelson JA (1998) Human cytomegalovirus persistently infects aortic ECs. *J Virol* 72:5661–5668.
14. Wang X, Huang DY, Huong SM, Huang ES (2005) Integrin $\alpha_v\beta_3$ is a coreceptor for human cytomegalovirus. *Nat Med* 11:515–521.
15. Wang X, Huong SM, Chiu ML, Raab-Traub N, Huang ES (2003) Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. *Nature* 424:456–461.
16. Feire AL, Koss H, Compton T (2004) Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. *Proc Natl Acad Sci USA* 101:15470–15475.
17. Muñoz-Chápuli R, Quesada AR, Angel Medina M (2004) Angiogenesis and signal transduction in ECs. *Cell Mol Life Sci* 61:2224–2243.
18. Serini G, Valdembri D, Bussolino F (2006) Integrins and angiogenesis: A sticky business. *Exp Cell Res* 312:651–658.
19. Baselga J (2002) Why the epidermal growth factor receptor? The rationale for cancer therapy. *Oncologist* 7(Suppl 4):2–8.
20. Tabernero J (2007) The role of VEGF and EGFR inhibition: Implications for combining anti-VEGF and anti-EGFR agents. *Mol Cancer Res* 5:203–220.
21. Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O (2003) Angiogenic and angiostatic factors in the molecular control of angiogenesis. *Q J Nucl Med* 47:149–161.
22. Jault F, *et al.* (1995) Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. *J Virol* 69:6697–6704.
23. Loffek S, Zigrino P, Steiger J, Kurschat P, Smola H, Mauch C (2006) Melanoma cell-derived vascular endothelial growth factor induces endothelial tubulogenesis within fibrin gels by a metalloproteinase-mediated mechanism. *Eur J Cell Biol* 85:1167–1177.
24. Smith MS, Bentz GL, Smith PM, Bivins ER, Yurochko AD (2004) HCMV activates PI (3)K in monocytes and promotes monocyte motility and transendothelial migration in a PI (3)K-dependent manner. *J Leukoc Biol* 76:65–76.
25. Yurochko AD (2008) in *Current Topics in Microbiology and Immunology: Human Cytomegaloviruses*, eds Stinski MF, Shenk T (Springer, Berlin), pp 205–220.
26. Johnson RA, Ma XL, Yurochko AD, Huang ES (2001) The role of MKK1/2 kinase activity in human cytomegalovirus infection. *J Gen Virol* 82:493–497.
27. Isaacson MK, Feire AL, Compton T (2007) Epidermal growth factor receptor is not required for human cytomegalovirus entry or signaling. *J Virol* 81:6241–6247.
28. Smith MS, Bentz GL, Alexander JS, Yurochko AD (2004) Human cytomegalovirus induces monocyte differentiation and migration as a strategy for dissemination and persistence. *J Virol* 78:4444–4453.
29. Boehme KW, Guerrero M, Compton T (2006) Human cytomegalovirus envelope glycoproteins B, H are necessary for TLR2 activation in permissive cells. *J Immunol* 177:7094–7102.
30. Wang D, Shenk T (2005) Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism *J Virol* 79:10330–10338.
31. Ryckman BJ, Jarvis MA, Drummond DD, Nelson JA, Johnson DC (2006) Human cytomegalovirus entry into epithelial and ECs depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. *J Virol* 80:710–722.
32. Siu LL, *et al.* (2007) Phase I/II trial of erlotinib and cisplatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck: A Princess Margaret Hospital phase II consortium and National Cancer Institute of Canada Clinical Trials Group Study. *J Clin Oncol* 25:2178–2183.
33. Lin MT, Yen ML, Lin CY, Kuo ML (2003) Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. *Mol Pharmacol* 64:1029–1036.
34. Zhang F, *et al.* (2004) Adenovirus E4 gene promotes selective EC survival and angiogenesis via activation of the vascular endothelial-cadherin/Akt signaling pathway. *J Biol Chem* 279:11760–11766.